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Effects of Temperature and pH on Survival of Free Nuclear Polyhedrosis Virus of 'Autographa californica'

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Effects of Temperature and pH on Survival of Free Nuclear Polyhedrosis Virus of *Autographa californica*

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The effects of temperature and low pH on replication and survival of nonoccluded *Autographa californica* nuclear polyhedrosis virus were investigated. No virus replication or formation of polynuclear inclusion bodies occurred at 37°C. The virus was immediately inactivated upon exposure to pH 2.0 and was inactivated within 1 h at pH 4.0. The virus titer slowly declined, a 3-orders of magnitude reduction in virus titer, at pH 5.0 during a 4-h exposure. Virus survival at pH 6.0 was equal to that of the control in cell culture medium 199 MK (pH 7.12).

Approval to release a microbial pest control agent depends on experimental results obtained after a tier testing protocol detailed in Subdivision M of the Pesticide Assessment Guidelines (1). This protocol requires that a variety of nontarget animals and plants be exposed to the microbial pest control agent and observed for signs of disease (death, illness, etc.). This is the classical approach to assessing pathogenicity of a microbe and is used to study disease in target species. However, a pathogenic microorganism placed in nontarget hosts may not multiply and produce disease. The lack of an endpoint(s) makes interpretation of test results and risk assessment difficult. Reasons for the failure of a pathogen to establish infection in a nontarget host might include nonpermissive body temperature or pH of gut. As an example, the mean body temperature of avians is 40 to 41°C and their gut pH is <3 in the crop and gizzard, rising to 7.0 in the lower intestine (15). On the other hand, insects are poikilotherms with an alkaline pH in the anterior gut (4).

The effect of the above optimum temperatures on the course of in vivo infection in insects by polyhedral inclusion bodies (PIBs) of nuclear polyhedrosis virus (NPV) has been reported by a number of investigators. For example, Ignoffo (7) found that NPV-infected *Heliothis zea* larvae held at 35°C incurred fewer mortalities than similar larvae held at optimum temperature (28°C). Morris (12a) showed that NPV-infected western hemlock loopers (*Lambdina fiscellaria luqubrosa*) had the greatest mortality at 21.5°C and that time to 50% mortality increased at higher temperatures. Complete inhibition of the infection cycle occurred at 35°C. Himeno et al. (5) showed that silkworms infected with NPV and incubated at 45°C for 1 or 2 h postinfection and returned to 25°C did not succumb to NPV disease. Exposure to the elevated temperature after inoculation was sufficient to inhibit virus adsorption. Kobayashi et al. (9) inoculated silkworm pupae (*Bombyx mori*) with NPV and studied the effect of various incubation temperature regimens. Infected pupae incubated at 35°C survived longer than controls held at 25°C. When the dosage of virus was lowered (10^{-2} or 10^{-3} dilution of original inoculum) and pupae were incubated at 35°C, the pupae escaped death from NPV infection. When infected pupae were incubated at 35°C and then transferred to 25°C at selected times, it was found that the longer the infected pupae were incubated at 35°C before transfer to 25°C, the longer they survived the infection. Johnson et al. (8) investigated the effect of higher incubation temperature on an NPV infection in the velvetbean caterpillar (*Anticarsia gemmatalis*). Temperatures above 32°C slowed the progress of the disease in the host. At 40°C, the disease did not develop at all in infected caterpillars.

Studies on the effect of hydrogen ion concentration on NPV have been limited to those pH values optimal for the dissolution of the PIBs. No information was found in the literature concerning the effect of pH on the survival of PIBs or the nonoccluded form of NPV.

This paper presents results of in vitro cell culture experiments on the effect of temperature and pH on survival of the nonoccluded form of *Autographa californica* NPV (ACNPV). Nontarget hosts would be susceptible to infections by only the free form of the virus since they likely would not possess the alkaline protease necessary for release of virus from PIBs. Temperature and pH effects were chosen for study because these two parameters could inhibit replication of virus in nontarget warm-blooded animals.

MATERIALS AND METHODS

Cell line and culture medium. The cell line used was derived from ovaries of *Trichoplusia ni* (cabbage looper) larvae and obtained from A. H. McIntosh (U.S. Department of Agriculture, Columbia, Mo.). It is an established cell line and was in its 20th to 30th passage during these experiments. The tissue culture 199 MK medium developed by McIntosh et al. (12) was used for cell propagation.

Cell transfer. The cells were maintained by weekly transfer into T25 plastic cell culture flasks (Falcon; Becton Dickinson Labware, Oxnard, Calif.) by 1:3 or 1:4 dilution. The medium was changed once between transfers. Cells for experiments and virus assays were grown in T75 plastic flasks by transferring 3- to 4-day-old cells from smaller flasks at a 1:3 dilution and were 5 days old at time of use.

Virus. ACNPV was obtained from A. H. McIntosh. Nonoccluded stock virus was prepared by harvesting infected cells after 3 days of incubation (28°C), grinding the cells in a glass tissue grinder, and centrifuging the suspension at 10,000 × *g* for 10 min. The supernatant containing the

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TABLE 1. Effect of incubation temperature on replication and survival of ACNPV NOV

Time (h)	Expt	$TCID_{50}$ at a temp (°C) of:			
		28[a]		37[a]	
		Infected cells	Virus only[b]	Infected cells	Virus only
24	1	$10^{3.86}$	$10^{4.3}$	$10^{3.5}$	$10^{4.0}$
	2	$10^{3.0}$	$10^{4.4}$	$10^{3.4}$	$10^{4.0}$
48	1	$10^{5.6}$	$10^{4.3}$	$10^{2.3}$	$10^{3.0}$
	2	$10^{5.4}$	$10^{3.9}$	$10^{3.3}$	$10^{3.8}$
72	1	$10^{6.5}$	$10^{4.1}$	$10^{3.6}$	$10^{3.5}$
	2	$10^{6.2}$	$10^{4.2}$	$10^{3.5}$	$10^{3.4}$
96	1	$10^{6.5}$	$10^{3.6}$	$10^{3.0}$	$10^{3.5}$
	2	$10^{6.5}$	$10^{3.5}$	$10^{3.2}$	$10^{3.8}$

[a] Average temperature during experiment: 28.0 = 27.99 ± 0.23°C; 37 = 37.06 ± 0.05°C.
[b] Virus suspended in cell culture medium.

nonoccluded virus (NOV) stage of ACNPV was saved and stored at −80°C in sterile 1.5-ml plastic tubes. Stock virus had a 50% endpoint titer of $10^{8.6}$ tissue culture infective doses ($TCID_{50}$) per ml when inoculated into *T. ni* cells (1a, 14). Titers were determined by averaging results of duplicate titrations.

Effect of temperature on growth of cell line. A stock of *T. ni* cells was grown in T75 cell culture flasks for 72 h. The cells were harvested and diluted 1:4 in fresh medium. Duplicate cell culture flasks (T25s) for each observation time and temperature were seeded with 5 ml of cell suspension and placed on a rocker platform for 1 h at 25°C. Flasks were incubated at 28, 35, or 37°C for 5 days. Cells were suspended from flask surfaces by shaking, 1-ml samples were diluted 1:3 in crystal violet solution (14), and cells were counted in a hemacytometer. Two counts were done on each flask, and the results are expressed as mean number of cells per milliliter.

Effect of temperature on virus growth. Two T75 flasks were seeded with cells at a 1:3 dilution in final volume of 25 ml and incubated at 28°C for 3 days. Culture medium was removed and replaced with an equal volume of fresh medium. The virus inoculum was added (0.1 ml of stock virus) and allowed to adsorb for 2 h at 25°C, after which medium and unadsorbed virus were removed and replaced with fresh medium.

At daily intervals, a 2-ml cell suspension was removed from each flask and centrifuged at 10,000 × *g* for 10 min. The pellet was suspended in an equal volume of sterile distilled water, and the PIBs were counted in a hemacytometer. Several 10-fold dilutions of the supernatant were assayed for NOV on *T. ni* cells in 96-well flat-bottom plates (Corning Glass Works, Corning, N.Y.) by the method of Brown and Faulkner (1a).

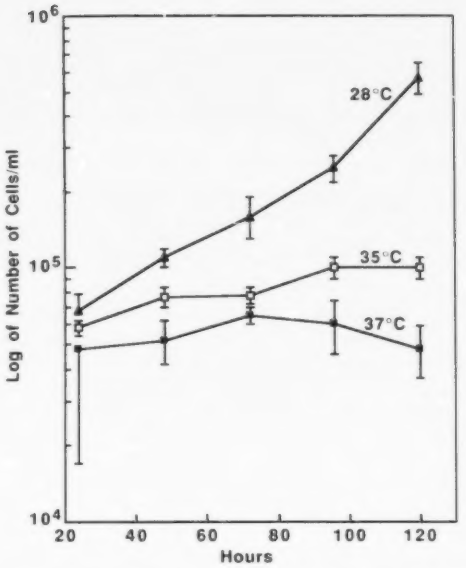

FIG. 1. Effect of incubation temperature on growth of *T. ni* cells. Vertical bars are standard errors of mean cell counts.

Virus preparation for pH study. A supernatant containing NOV was prepared as above. The NOV was sedimented from the supernatant in a Beckman SW60 Ti swinging-bucket rotor by centrifugation in a Beckman L2-65b ultracentrifuge at 144,000 × *g* for 2 h. The supernatant fluid was drained from the tubes and discarded. The virus pellet was suspended in one of four buffers (pH 2, 4, 5, or 6) or in tissue culture medium 199 MK by pipette. Samples (0.2 ml) were removed at 0, 1, 2, and 4 h after exposure and diluted 10-fold in medium (199 MK). Virus content was determined by $TCID_{50}$ endpoint dilution (14).

Buffers. Acidic buffers corresponding to pH 2, 4, 5, and 6 were chosen to cover the pH range of the avian alimentary canal, simulating what the virus would be exposed to after oral inoculation. The buffer for pH 2.0 consisted of 0.1 M glycine plus 0.1 M HCl; that for pH 4 was 0.1 M citrate plus 0.1 M HCl; that for pH 5 was 0.2 M sodium acetate plus 0.2 M acetic acid; and that for pH 6.0 was 0.1 M KH_2PO_4 plus 0.1 M NaOH (2, 10). The pH of the buffer was measured with

TABLE 2. Effect of hydrogen ion concentration (pH) on survival of NOV of ACNPV

Time (h)	Replicate in each expt	Surviving virus ($TCID_{50}$) at pH of:							
		7.12[a]		6.0		5.0	4.0		2.0
		Expt A	Expt B	Expt A	Expt B		Expt A	Expt B	
0	A	$10^{5.6}$	$10^{5.8}$	$10^{5.5}$	$10^{5.6}$	$10^{5.3}$	$10^{3.8}$	$10^{3.8}$	0
	B	$10^{5.5}$	$10^{5.3}$	$10^{5.9}$	$10^{5.3}$	$10^{5.4}$	$10^{3.5}$	$10^{3.5}$	0
1	A	$10^{5.6}$	$10^{5.6}$	$10^{5.3}$	$10^{4.6}$	$10^{4.3}$	0	0	0
	B	$10^{5.9}$	$10^{5.5}$	$10^{5.4}$	$10^{4.7}$	$10^{5.6}$	0	0	0
2	A	$10^{5.5}$	—[b]	$10^{5.4}$	$10^{5.6}$	$10^{2.8}$	0	0	0
	B	$10^{5.9}$	—	$10^{5.5}$	$10^{5.4}$	$10^{2.5}$	0	0	0
4	A	$10^{5.5}$	$10^{5.6}$	$10^{5.5}$	—	$10^{2.5}$	0	0	0
	B	$10^{5.9}$	$10^{5.5}$	$10^{5.6}$	—	$10^{2.2}$	0	0	0

[a] Cell culture medium.
[b] —, Samples not assayed.

an Orion model 601A pH meter after standardization with pH 7 and 4 buffers.

RESULTS

Incubation of *T. ni* cells at a temperature above optimum affected their growth. Comparison of growth curves at 28, 35, and 37°C (Fig. 1) showed that higher temperatures, especially 37°C, slowed growth of cells.

Production of NOV at the optimum temperature of 28°C followed a normal growth cycle, reaching a peak of $10^{6.2}$ to $10^{6.5}$ $TCID_{50}$ at 72 h (Table 1). Virus production in infected cells incubated at 37°C was inhibited. The higher incubation temperature was not lethal to the virus as seen by a constant titer of virus incubated in cell-free medium (Table 1). Enumeration of PIB formation at the two temperatures showed that at 37°C no PIBs could be counted (data not shown) but that at 28°C there was a steady increase in numbers during the 96-h experiment (1.3×10^6 to 6.2×10^8 PIBs per ml).

The NOV did not survive exposure to pH 2; the zero time assay was below detectable limits ($<10^{-1}$) (Table 2). Exposure of virus to pH 4.0 caused an approximate 1 to 2 long drop in virus survival at zero time when compared with the control, and after 1 h of exposure, no viable virus was found. Results of pH 5.0 exposure indicated a 1 log decrease at 1 h and a 3 log decline by 2 h with little change during the next 2 h of exposure (4 h). Virus survived at pH 6.0 and in culture medium at pH 7.12.

DISCUSSION

Review of the literature revealed that replication of NPVs of most insects is inhibited by incubation of infected hosts at or above 35°C. The results of the present research show that temperature inhibition also occurs for the nonoccluded form of an insect virus, ACNPV. The virus did not die at 37°C, but maintained a constant titer in *T. ni* cell cultures incubated at 37°C for 96 h. Incubation of virus in cell-free medium at the same temperature showed a similar maintenance of titer with little decline ($10^{4.0}$ to $10^{3.5}$ in 96 h); additionally, no PIBs were formed in cultures at 37°C. It would appear, therefore, that the higher temperature restricted the replication of the virus after the infection was initiated, since virus adsorption occurred at a permissive temperature (25°C) with a multiplicity of infection high enough to ensure that all cells were infected. The virus inhibition at higher temperatures may be due to the slower growth of the cells, because during the 96 h of incubation the cell numbers only increased 10-fold (10^5 to 10^6). Virus replication requires enzymes from actively dividing cells (e.g., DNase, transcriptase, mRNA), any one of which may be temperature sensitive. Cytopathological changes have been observed in nuclei of infected cells in vitro during the eclipse phase of the viral replication cycle of baculoviruses (4), suggesting some form of cellular involvement.

Exposure of the NOV of ACNPV to low pHs (≤7) showed that pH 2 and 4 are lethal to the virus. Therefore, if a bird or mammal was inoculated orally with NOV, the virus would become inactivated by gut pH (<3). Oral inoculation of vertebrates with PIBs also is unlikely to result in viral replication, since they lack the alkaline protease necessary to dissolve the PIBs into the infective free-virus form (4). Previous studies have suggested that PIBs ingested by avians are shed in the feces without replicating (3, 6, 11).

Based on the results presented here and previous observations (3, 6), it seems likely that safety testing of pathogenicity of NPVs in endothermic animals will produce negative results, i.e., no viral replication owing to relatively high body temperatures. Additionally, the viricidal effect of the low pH in the stomach or crop and proventriculus provides another barrier to successful viral replication in birds.

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